In Vitro Cell. Develop. Biol., in Press (1997)

SCIENTIFIC REPORT

A SIMPLIFIED METHOD FOR TISSUE ENGINEERING SKELETAL MUSCLE ORGANOIDS IN VITRO

JANET SHANSKY[1], MICHAEL DEL TATTO[1], JOSEPH CHROMIAK[1] and HERMAN VANDENBURGH[1,2]

[1]Department of Pathology, Brown University School of Medicine and The Miriam Hospital, Providence, RI; [2]Department of Mol. Pharm. and Biotech., Brown University, Providence, RI 02906

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Correspondence to: Dr. Herman Vandenburgh

Dept. Path., Brown University/The Miriam Hospital

164 Summit Ave.

Providence, RI 02906

Tel: 401-331-8500 Ext. 4273 FAX: 401-751-2398

E-mail: Herman_Vandenburgh@brown.edu.

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Dear Editor:

Tissue-engineered three dimensional skeletal muscle organ-like structures have been formed *in vitro* from primary myoblasts by several different techniques (3,4,13). The resulting "organoids" display many of the characteristics of *in vivo* muscle including parallel arrays of postmitotic fibers organized into fascicle-like structures with tendon-like ends. They are contractile, express adult isoforms of contractile proteins, can perform directed work, and can be maintained in culture for at least 4 weeks. The *in vivo*-like characteristics and durability of the muscle organoids make them an ideal model system for long term *in vitro* studies on mechanotransduction mechanisms and on muscle atrophy induced by decreased muscle tension (1,8,11,12). Tissue engineered skeletal muscle organoids have also been used as an implantable device for the systemic delivery of recombinant proteins (7).

Current methods for muscle organoid formation are limited by the number which can be formed at one time, and by the complicated procedures necessary to induce organogenesis. Induction of muscle organogenesis from skeletal myoblasts utilizes either internally or externally generated mechanical tension as an important element for the alignment of the fusing myoblasts and subsequent organoid formation. For example, a computerized mechanical cell stimulator device provides external longitudinal mechanical forces during the first 2-3 days of myoblast proliferation to orient the developing myofibers parallel to each other and to the direction of stretch (9). After 6-10 days of additional complex mechanical stimulation, the edges of the cell monolayer roll in to form an organ-like structure (13). Similar muscle organogenesis has been induced passively by plating

high density monolayers of avian myoblasts onto a collagen-coated, flexible plastic substrate held in place by stainless steel pins (3,4). After 10-15 days of culture, the differentiated myofibers begin twitching spontaneously, generating internal tensions which cause the cell sheet to detach from the flexible substrate while remaining held in place by the stainless steel pins. While these methods have been useful for small-scale muscle organogenesis studies, they have not been scaled up to provide the large number of samples necessary for more complex studies. In this report, we describe a simple method for generating large numbers of muscle organoids from either primary embryonic avian or neonatal rodent myoblasts.

Myoblasts are plated into flexible, rectangular culture wells (20mm long x 10mm wide x 10mm deep) constructed from transparent 0.01 inch thick silicone rubber sheeting (Silicone Speciality Fabricators, Inc., Paso Robles, CA). A cardboard cutting pattern sheet containing approximately 50 well outlines (Figure 1A insert) is placed under three 20 cm x 28 cm silicone rubber sheets, and the well edges cut with a scapel. Wells are formed from the silicone membrane by folding the middle flap at each end up and the two outside flaps in and gluing with RTV silicone sealant (General Electric Co, Waterford, NY) applied with a 10 ml syringe. After gluing into the three dimensional shape, pieces of chemically cleaned stainless steel screening (9) 5 mm wide x 10 mm long, and bent 90° in the middle, are glued to the bottom of the culture wells at each end with the RTV silicone sealant (Figure1A). The screening provides a surface to which the cells can attach as they proliferate. Several hundred wells can be constructed in several days and the wells can be reused 2-3 times before they begin to leak.

The wells are secured to notched aluminum brackets (Figure 1B) using stainless steel screws and washers placed through small holes cut in the ends of the wells. Each bracket can hold up to 12 culture wells, and consists of 2 square bars (1 cm x 1 cm x 20 cm) with twelve 3 mm wide by 6 mm deep notches on the top. The brackets are connected at their ends by slotted flat bars (10.5 cm x 1.2 cm) which allows the distance between notched bars to be adjusted. The wells are placed into the bracket notches and the screws tightened to hold the wells in place. For optimal formation of organoids, the bar spacing is set at 30 mm; this stretches the wells approximately 50% from their resting length of 20mm. After the wells are attached to the brackets and stretched, they are sprayed with 6-8 coats of 0.1% (w/v) Type 1 collagen (Collaborative Biomedical Products, Bedford MA) using an airbrush or chromatographic sprayer (10). They are placed in tissue culture trays (USA Scientific Plastics, Ocala, FL), and sterilized with ethylene oxide; they can also be autoclaved if desired. The sterile wells can be stored at room temperature for 2 - 3 weeks. On the day of culture, the wells are preincubated with Earle's Balanced Salt Solution (EBSS) for several hours before cell plating.

Avian pectoralis myoblasts (5) or neonatal rat myoblasts (2) are isolated and plated using normal tissue culture techniques. For the avian muscle cells, plating density is 5 to 7.5 x 10^6 /well in 1 mL of 85/10/5 growth medium [Basal Medium Eagles containing 10% horse serum, 5% chicken embryo extract, penicillin (100U/mL), and amphotericin B (5µg/mL)]. Unless otherwise noted, all tissue culture reagents are purchased from Sigma Chemical Co., St. Louis, MO. Cultures are fed daily with fresh 85/10/5. Avian myoblasts proliferate and fuse into multinucleated myotubes beginning 48 h after plating, align

parallel to the direction of substratum tension, and become striated and contractile by 96-120 h. Approximately 5 days after plating, the cell layer lifts off the bottom of the silicone rubber wells, (while remaining attached to the screens at both ends), and the long edges of the cell layer roll in to form a muscle organoid (Figure 2A) similar to those formed using more complex equipment (13). These organoids contain organized and contractile myofibers (Figure 2B).

Mammalian organoids can be tissue engineered from primary neonatal rodent myoblasts using a similar technique but require the addition of a Matrigel™ - collagen solution to the cells. Myoblasts are isolated from the forelimbs and hindlimbs of rat neonates following the method of Rando and Blau (2). Isolated primary cells are suspended in a 1:6 solution of Matrigel™ (Collaborative Biomedical Products) : collagen (Type 1, 1.6mg/mL) prepared with growth medium [GM: Ham's F-10 containing 20% fetal bovine serum, and 2.5 ng/mL bFGF, penicillin (100U/mL) and streptomycin (50 U/mL)], and plated into the wells at a concentration of 4 $x10^6$ cells/ 0.750mL. The wells are placed in a 37°C incubator for 2-6 h to allow the Matrigel™ - collagen mixture to gel before carefully overlaying with 1ml GM. Cultures are maintained in GM for 3 days, fusion medium [DMEM (high glucose) with 10% horse serum + penicillin] for 3 days, and maintenance medium [DMEM (high glucose) with 10% horse serum, 5% FBS + penicillin) for up to 4 weeks. The gel:cell mixture condenses and dehydrates during the first 2-3 days, pulling off the elastic substratum and generating internal tensions to align the forming myofibers (Figure 2C). The extracellular matrix gel required for mammalian but not avian organoid development may be due to the greater proliferative ability of connective tissue forming fibroblasts in the

latter than the former cultures (unpublished observation). Myoblast cell lines such as C2C12 which are devoid of fibroblasts can also be formed into organoids, but also require the Matrigel™ - collagen mixture (data not shown).

The differentiated muscle organoids in the elastic wells can be removed from the brackets without tension release and transferred to other tissue culture chambers for long term muscle growth/atrophy studies. This is accomplished by placing a stainless steel screen "spacer" 1 cm wide and the length of the culture well into the wells just above the organoid. The wells can then be removed from the brackets by slipping the well screws out of the top of the bracket notches. Using this simple technique, avian muscle organoids have been removed from the brackets, transferred to modified bioreactor cartridges of a continuous perfusion system (CELLCO, Inc., Germantown, MD) and maintained without myofiber atrophy for up to three weeks in DMEM with 2.5% (v/v) horse serum (6).

In summary, these procedures offer several advantages over previous techniques for muscle organoid formation. No mechanical stretching apparatus is needed, the procedures are simplified, and large numbers of uniform, *in vivo*-like organoids are easily formed and transferrable to other tissue culture environments. These new techniques should prove useful in studies where large scale skeletal muscle organoid culturing is required.

(Supported by NASA Grants NAGW-4674 and NAG2-914)

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FIGURE LEGENDS

Figure 1. Rectangular culture wells are formed from silicone rubber sheeting and secured to aluminum brackets. Wells measuring 20mm long x 10mm wide x 10mm deep are glued from a precut thin silicone rubber sheet (insert) and formed into three dimensional wells (A). Stainless steel screening at each end of the boat provides a surface for cell attachment. Wells are secured to notched aluminum brackets using stainless steels screws and washers (B). Each bracket holds 12 culture wells and the spacing between the two notched aluminum bars is adjustable. Bars represent 10 mm and 25 mm in (A) and (B), respectively.

Figure 2. Skeletal muscle organoids contain parallel arrays of myofibers expressing sarcomeric tropomyosin. Avian and mammalian myoblasts are grown and maintained in the culture wells as described. After 14-18 days in culture, muscle organoids (A) are fixed, and stained with an antibody against sarcomeric tropomyosin (Sigma Chem Co., St. Louis MO), followed by incubation with an avidin-biotinylated secondary antibody coupled to horse radish peroxidase (Vectastain, Vector Laboratories, Burlingame CA), and development with diaminobenzidine to form a dark precipitate. (B) avian skeletal muscle organoid, whole mount stained, showing aligned surface myofibers; (C) mammalian organoid formed from neonatal rat skeletal myoblasts with myofiber alignment in the long axis of the organoid. Bars represent 10 mm, 200 μM and 50 μM in (A), (B), and (C), respectively.

FIGURE 1

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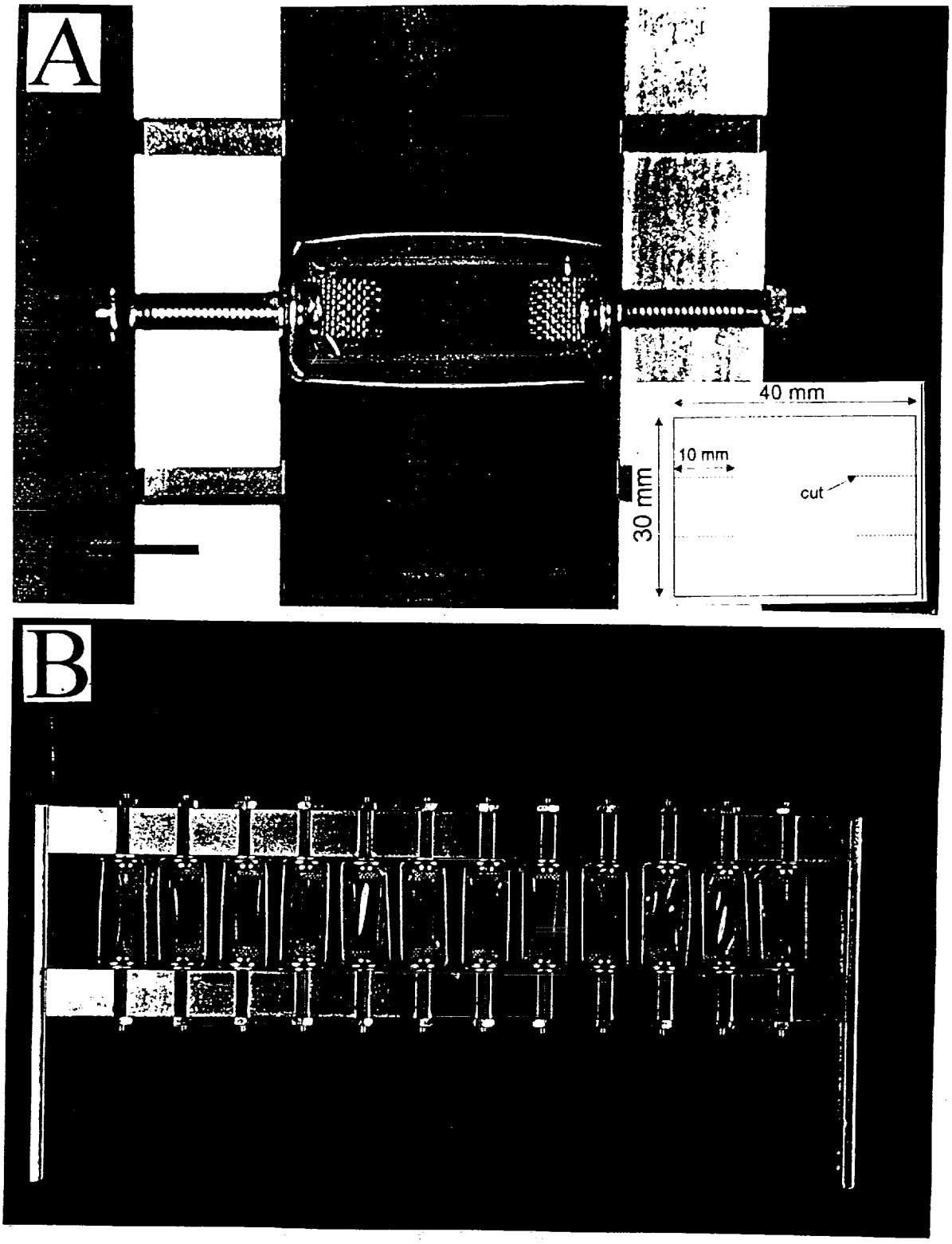

FIGURE 2

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